# Probing Heme Trafficking Factors via Organellar Contact Points Using Genetically Encoded Fluorescent Heme Sensors

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By

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**Probing Heme Trafficking Factors via Organellar Contact Points Using Genetically Encoded Fluorescent Heme Sensors**

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## TABLE OF CONTENTS

## ABSTRACT

Heme is an important protein cofactor and signaling molecule that plays diverse roles in biological systems. The hydrophobicity and cytotoxicity of heme necessitates that it is transported and trafficked in a regulated manner. However, the molecules and mechanisms responsible for mediating heme trafficking remain poorly understood. Until recently, the tools to study heme *in vivo* did not exist, but the emergence of genetically encoded fluorescent sensors has enabled comprehensive real time analysis of heme in model organisms such as *Saccharomyces cerevisiae*. This study showcases a new a protocol that allows investigation of heme trafficking from its site of synthesis in the matrix side of the mitochondrial inner membrane to the outer matrix, cytosol, and nucleus over time. The method allows for the simultaneous examination of heme re-population in three cellular compartments after chemically depleting it. The study revealed that mitochondrial contact points play central roles in regulating heme availability and illuminates novel approaches to heme trafficking. These methods have the potential to be adapted to more inclusive compartmental analyses and enable a better understanding of heme trafficking which can empower innovative approaches to study infectious diseases, neurodegenerative disorders, and anemias associated with perturbations in heme cellular dynamics.

## INTRODUCTION

Heme is an essential signaling molecule and protein cofactor integrated in many cellular pathways and mechanisms such as: gas transport, electron transfer, chemical catalysis, energy metabolism, etc.[1] Heme synthesis is split between the mitochondria and cytosol, with the first and last three steps occurring in the mitochondria.[1] A typical heme molecule comprises of a hydrophobic protoporphyrin ring that chelates an iron (Fe) core via four nitrogen molecules. Despite its essential presence in cells, the metallic core makes heme cytotoxic— raising questions concerning the manner of heme trafficking and mobilization in cell, since both processes must be closely regulated and strictly controlled.[2]

Heme exists in two "pools" within the cell: inert heme tightly bound to high affinity hemeproteins, and labile heme used by low affinity hemeproteins primarily for signaling, and thus in constant flux between a bound an unbound state.[3] Therefore, labile heme is an ideal target for analyzing heme dynamics and regulation. The Reddi lab at Georgia Tech recently developed two families of genetically encoded fluorescent sensors that enable the detection, quantification, and imaging of labile heme within *Saccharomyces cerevisiae* (baker's yeast) cells.[1] These are tri-domain sensors with the following protein encoded domains: a heme binding domain (Cyt $b_{562}$) with no fluorescent activity), a green fluorescent protein (eGFP), and a red fluorescent protein (mKATE)—to allow for the measurement of bioavailable heme. As Cyt $b_{562}$ binds to labile heme, the fluorescence of eGFP quenches via a Forster resonance energy transfer where the excited green chromophore transfers energy to the red chromophore via dipole-dipole coupling without changing the overall fluorescence of mKATE, allowing a ratiometric quantification of heme. One family of the sensors is tight binding with a high affinity for heme (100% bound) and

the other is weaker binding with a lower affinity for heme (30-50% bound) —optimal for observing variations in heme concentrations over time.[1]

Previous studies have implicated physical organelle contact points in lipid trafficking.[4] Heme can actually be modeled as a lipid due to its hydrophobic core, which has similar chemical properties to fatty acids and poly-glycerides.[4] This study describes a new method to study heme kinetics and uses it to identify key contact points between the mitochondria and proximal organelles involved in heme trafficking. The mitochondria are a target because all intra-cellular heme stems from them and must be transferred to the other cellular compartments that require it, the mitochondria are also extremely important and closely associated with the ER. Ungermann and colleagues' have classified various sets of contact points, of which two were of primary interest in context of this study: the endoplasmic reticulum-mitochondria encounter structure (ERMES), and the vacuole-mitochondrial patch (vCLAMP). The ER wraps around the nucleus and provides a potential pathway for heme trafficking from the mitochondria, meanwhile certain vacuolar contact points have been shown to rescue cellular defects from mitochondrial and ER contact point mutants.[5] Therefore, five of these contact point knockout mutants were studied via the time dependent heme saturation studies. Three ERMES contact points: Gem1, Mdm34, and Mdm12, and two vCLAMP contact ponts: Ypt7, and Vps39.

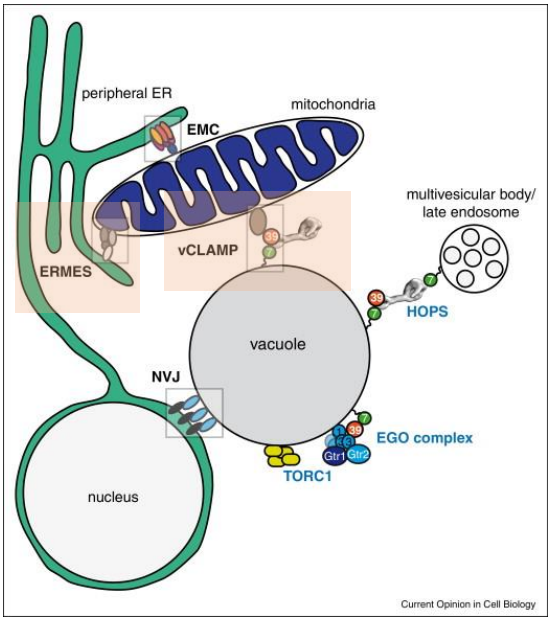


**Figure 1.** Cellular contact points as identified by Ungermann and colleagues, with a special emphasis on ERMES and vCLAMPS for the purposes of this study

## METHODS AND MATERIALS

The guiding principle for this study was to identify genes of interest that might be important for heme trafficking and study heme kinetics in *S. cerevisiae* strains that lacked those genes (knockouts). Genes of interest were primarily obtained via literature searches, they were then matched with samples in the commercially avaialble *S. cerevisiae* knockout library via the yeast genome database. Those strains were isolated from the knockout library, transformed with the ratio metric fluorescence sensors, and heme kinetics experiments were performed to determine whether the gene had any significance in heme trafficking.

### *Sample Preparation*

Cell strains of interest were obtained from the *Saccharomyces cerevisiae* gene knockout collection and grown on YPD + G418 (antibiotic) plates overnight to select for the knockout. Certain colonies were isolated and re-patched on YPD G418 plates, which were then used to create cells stocks (in 20% glycerol YPD) and stored in -80$^{o}$C.

### *Transformation*

All strains of interest were transformed individually with three plasmids, each localizing the sensor to a certain cellular compartment—the cytosol, the mitochondria, and the nucleus—creating three separate strains with one localized sensor each.

The transformations were performed by growing cell strains from the knockout stock in 10mL of liquid YPD media overnight. The following day, the optical densities (OD) of each strain were measured using absorbance spectroscopy and the volume of 0.1 M lithium acetate needed were calculated using the following equation:

$$\left(\frac{10{,}000\mu L * OD * 2x10^{7}}{2x10^{9}}\right) = \mu L\ of\ 0.1M\ Lithium\ Acetate$$

The cells were centrifuged for five minutes and the supernatant discarded. They were resuspended in the appropriate volume of lithium acetate and transferred to a microcentrifuge tube, from which 50 μL were transferred to microcentrifuge tubes labeled with the appropriate plasmid or $H_2O$ (negative control). The tubes were centrifuged for 1 minutes and the supernatant removed using a 100 μLpipette without disturbing the pellet.

The following were added to each tube without resuspending the pellet: 240 μL of 50% PEG, 36 μL of 1 M lithium acetate, 10 μL of ssDNA, 49 μL of DI water, and 1 μL of the appropriate plasmid or DI water (blank). The tubes were vortexed until all the solvents and the pellet were completely mixed and homogenized. The samples were heat shocked at 30$^{o}$C for 30 minutes and 42$^{o}$C for 20 minutes. They were then centrifuged for 3 minutes at 4$^{o}$C and the supernatant was removed.

The pellets were resuspended in 500 μL of DI water and 100 μL of each was plated on SC-Leu plates with glass beads. The plates were shaken for 2 minutes, the beads discarded, and then the plates were grown for 48 hours before colonies were selected for twice and freezer stocks prepared.

***Heme Trafficking Kinetics Protocol***

For each kinetics experiment, an empty vector (yeast strain with the plasmid but without the sensor), a wild type (WT) strain with each of the localized sensors, the knockout of interest with each of the localized sensors, and hem1Δ strain (produces no heme), were plated on SC-LEU or SC-LEU+ALA (hem1Δ strain) plates.

Once the cells grew, a patch was resuspended in 1000 μL of DI water and their OD's were measured in an Agilent Technologies Cary 60 UV-Vis reader. The WT cells and the knockout cells were split into normal or plus succinyl acetone (a heme synthesis inhibitor). The

empty vector and normal WT cells were grown with an initial density (ID) of 0.01, while the hem1Δ, plus succinyl acetone (+SA) WT, and normal knockouts were grown with an ID of 0.02, and the +SA knockouts were grown with an ID of 0.03. The following calculation was used to determine the volume of each cell sample needed to grow them at the appropriate ID in 5000 µL of SCE-Leu media:

$$volume = \left(\frac{ID * 5000\ \mu L}{OD}\right)$$

In appropriately labeled tubes, 5 mL of media was dispensed, and the respective volume of cells was added. In the +SA tubes, enough succinyl acetone was added to have a 500 µM concentration. The cells were incubated overnight for around 14-16 hours.

The following day, the OD's were measured, and the appropriate volumes of the cells needed to create a 1 OD solution was transferred to a fresh tube. The cells were pelleted, washed with DI water twice, and resuspended in 1mL of SC-LEU media. The +SA groups were split into ++SA and +-SA. The ++SA groups received succinyl acetone again (500mM) whereas the +-SA did not. The cells were plated in duplicates in a black 96 well plate and fluorescence measurements were taken over the course of four hours in a Synergy Mx, BioTek plate reader.

Once the data was collected, it was analyzed to obtain the eGFP/mKATE ratio and percent heme bound was calculated by using the ratio of the ++SA groups and the +-SA groups. Before any calculations, the empty vector data was used to account for background fluorescence. The percent heme bound for the WT, gene knockout of interest, and hem1Δ were compared.

***Heme Kinetics for heme and depletion***

Wild type cells with HS1 sensors in each compartment were prepared in the same manner as the normal heme kinetics studies. However, instead of growing them overnight in succinyl acetone, the cells were separated in 6 different Erlenmeyer Flasks, 2 for each compartment. One

OD of cells were placed in each flask with SC-Leu media, and for each pair only one received succinyl acetone, while the other was not treated with anything. Every hour, an aliquot of the samples would be removed, centrifuged, washed once with DI water, normalized to 10 OD and the fluorescence measured. This was performed for a total of seven hours.

# RESULTS

## *Heme Kinetics Saturation and Depletion*

In order to be able to use the kinetics protocol to study heme trafficking from each of the compartments, we first had to verify that the system was operating in the expected manner. We did this by using the kinetics protocol to observe the saturation of heme, and a slightly modified protocol to observe the depletion of heme in all three compartments in wild type cells. Figure 2. shows the saturation of heme in the cells under the normal heme kinetics conditions. Four hours is a sufficient time for the cells to saturate at least to obtain 50% bound heme. Figure 3. shows the depletion of heme in the modified kinetics experiment over the course of 8 hours. Although four hours is sufficient to deplete heme to only 20% bound, it takes 1 to 2 hours longer to deplete it 100% (0% bound) depending on the compartment.

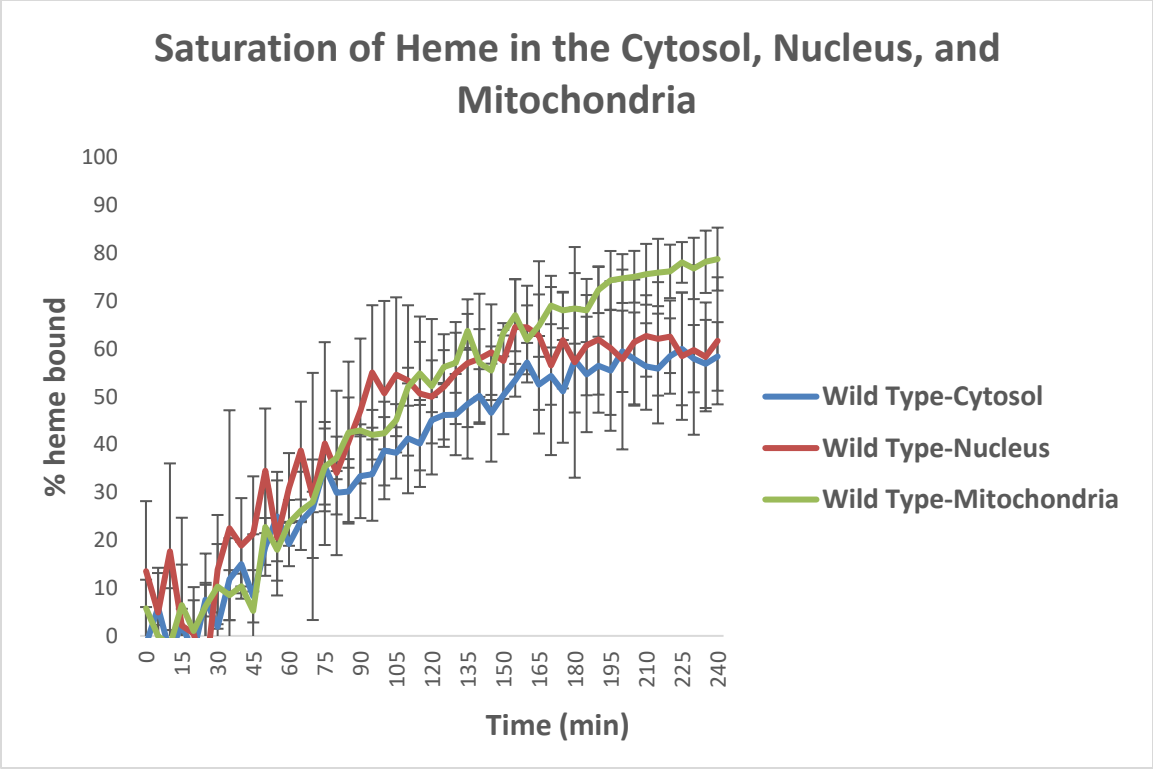


**Figure 2**. *Saturation of Heme in the Cytosolic, Nuclear, and Mitochondrial sensors.* WT cells transformed with the cytosolic (blue), nuclear (red), and mitochondrial (green) high affinity sensors were grown overnight with succinyl acetone to inhibit heme synthesis. After growing to approximately 1 OD the cells were plated in fresh SC-Leu media without succinyl acetone to observe their re-saturation with heme fluorescently over the course of three hours. The nucleus cytosol and the nucleus reached 60% heme saturation, and the mitochondria saturated to about 80%.

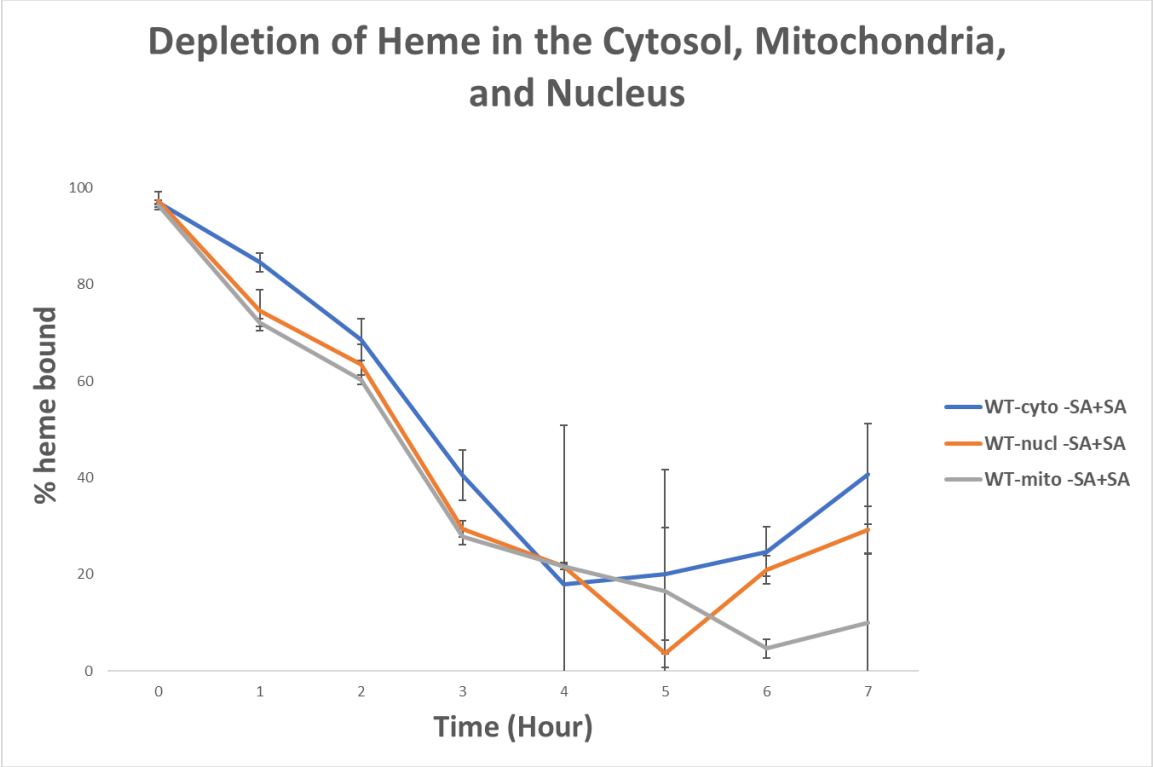


**Figure 3**. *Depletion of Heme in the Cytosol, Mitochondria, and Nucleus.* WT cells transformed with the cytosolic (blue), nuclear (orange), and mitochondrial (grey) high affinity sensors were grown overnight without succinyl acetone and were treated with in the morning and incubated for 7 hours, with fluorescent reads once an hour. The cytosol's lowest heme bound was 20%, the nucleus' was nearly 0%, and the mitochondrial 5%.

***Heme Kinetics of ERMES contact points***

The three ERMES contact points studied were Gem1 (Figure 4.), Mdm12 (Figure 5.), and Mdm34 (Figure 6.). Gem1 is a GTPase implicated in phospholipid transfer, and important for mitochondrial morphology.[6] The heme kinetics assay for *gem1Δ* showed increased nuclear trafficking (Figure 4.). Mdm12 is an outer membrane protein necessary for the movement of mitochondria to daughter cells[7], the heme kinetics assay for *mdm12Δ* showed increased nuclear trafficking as well (Figure 5.). Mdm34 has been implicated in calcium and phospholipid transport and is co-localized with the peroxisomes,[9] the *mdm34Δ* also showed increased nuclear trafficking (Figure 6.)

The *gem1Δ* cells nucleus integrated heme at a significantly higher rate and to a higher degree (85%) when compared to WT's nucleus (40%) (Figure 4.) Similar reuptake differences were observed for *mdm12Δ* (90% for ko and 50% for WT) and *mdm34Δ* (90% for ko and 60% for WT) (Figure 5. and Figure 6.).

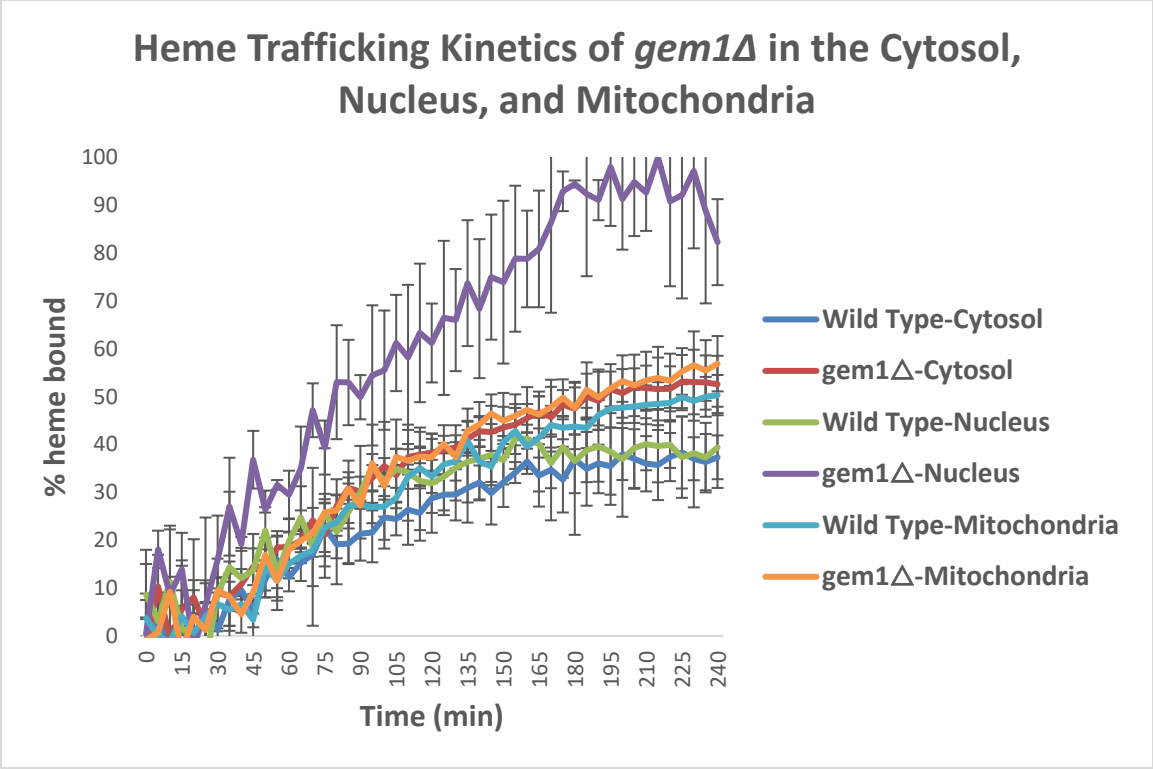


**Figure 4.** *Heme Kinetics of Gem1Δ in the Cytosol, Nucleus, and Mitochondria.* The heme saturation in Gem1 knockouts for each of the compartments was fluorescently measured over the course of four hours along with WT's under the same conditions.

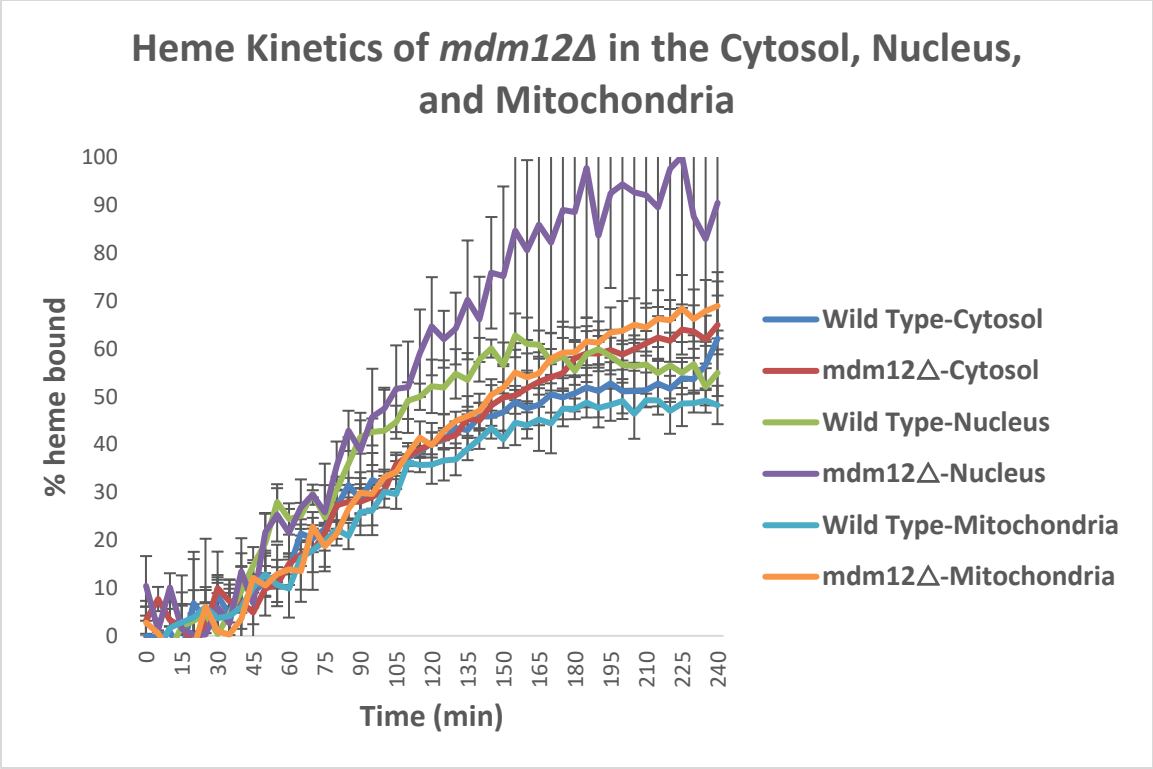


**Figure 5.** *Heme Kinetics of Mdm12Δ in the Cytosol, Nucleus, and Mitochondria.* The heme saturation in Mdm12 knockouts for each of the compartments was fluorescently measured over the course of four hours along with WT's under the same conditions.

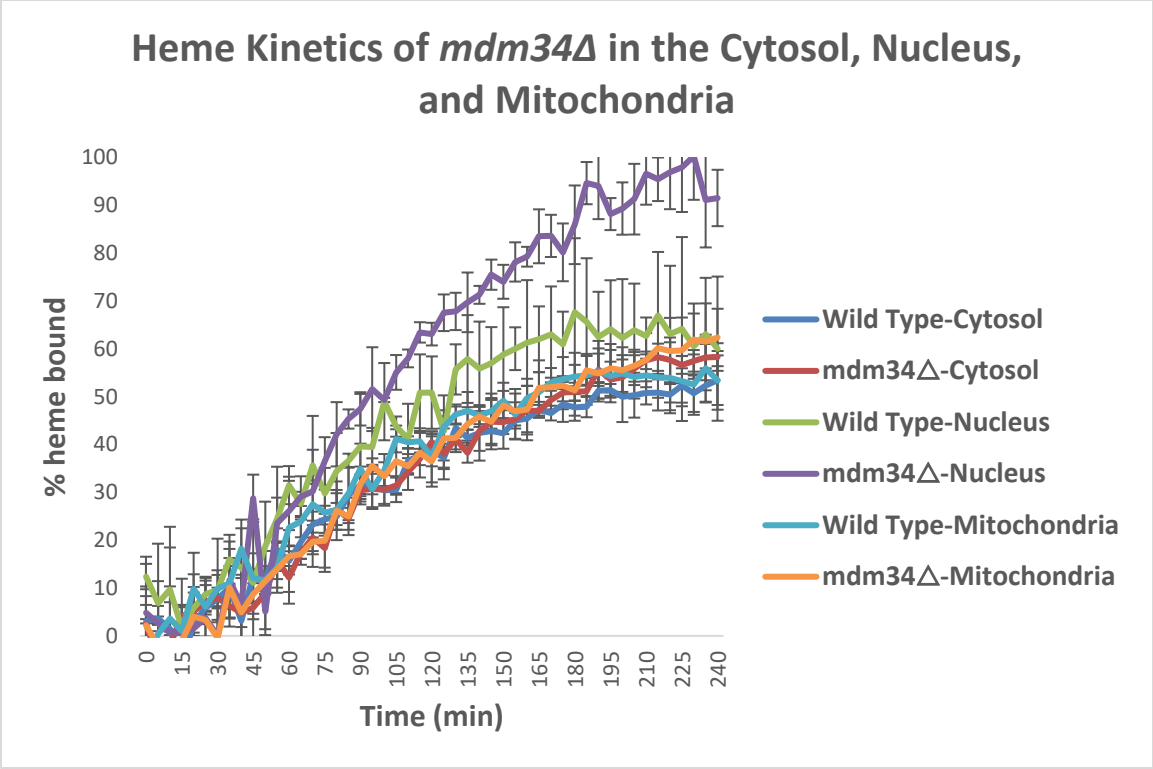


**Figure 6.** *Heme Kinetics of Mdm34Δ in the Cytosol, Nucleus, and Mitochondria.* The heme saturation in Mdm34 knockouts for each of the compartments was fluorescently measured over the course of four hours along with WT's under the same conditions.

***Heme Kinetics of vCLAMP contact points***

The following two vCLAMP contact points were studied: Ypt7 and Vps39. Ypt7 is a GTP binding protein that is needed for homotypic fusion during vacuole inheritance. It also interacts with Vps 39, a guanine nucleotide exchange factors implicate in membrane fusion, and a cofactor for both protein and lipid trafficking.[8] The *ypt7Δ* data is shown in Figure 7. and the *vps39Δ* data is shown in Figure 8.

Unfortunately, the *ypt7Δ* data showed a higher degree of error and the WT for the nucleus started with 50% heme bound. However, the ko nucleus does get saturated to a higher degree than the other WT compartments, and so the ko mitochondria, to a lesser degree (Figure 7.). The *vps39Δ* data show little to no trafficking to the nucleus (Figure 8.). The vCLAMP kinetics results were not nearly as cohesive as the ERMES results, but certain abnormalities in trafficking were still identified. The *ypt7Δ* appears increases heme trafficking to the nucleus similar the ERMES (Figure 7.), and *vps39Δ* inhibits trafficking to the nucleus (Figure 8.)

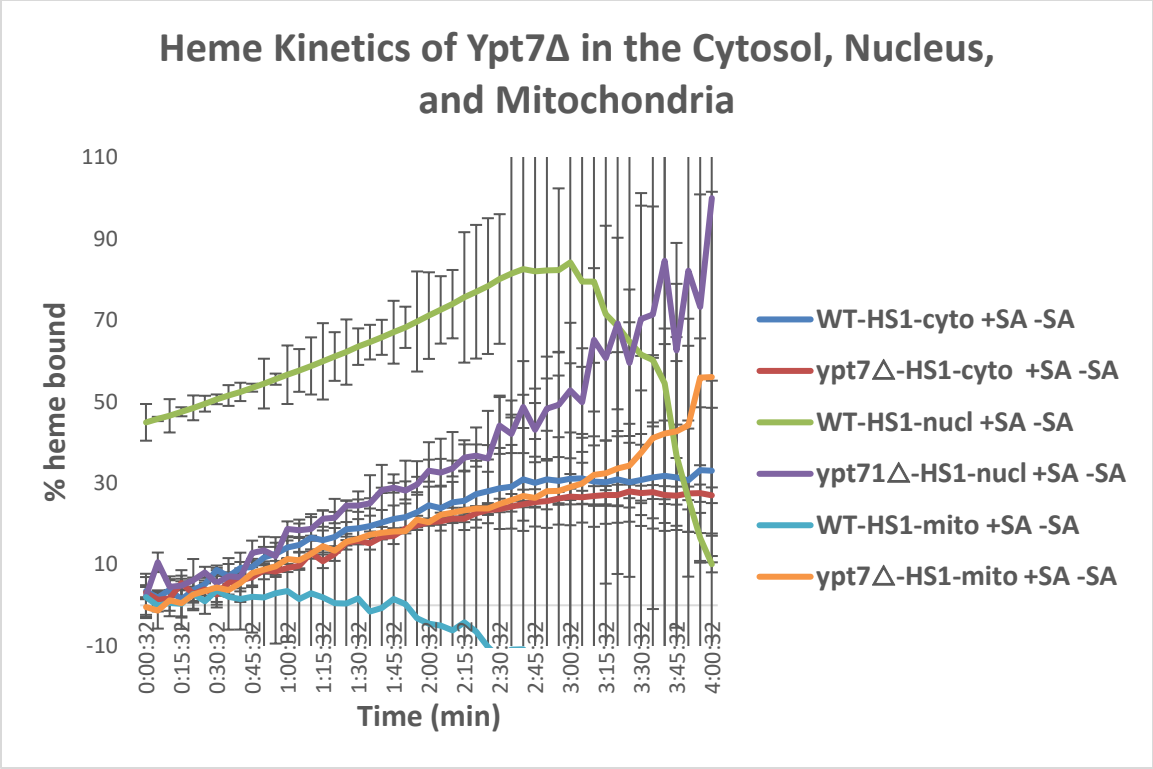


**Figure 7.** *Heme Kinetics of Ypt7Δ in the Cytosol, Nucleus, and Mitochondria*. The heme saturation in Ypt7 knockouts for each of the compartments was fluorescently measured over the course of four hours along with WT's under the same conditions.

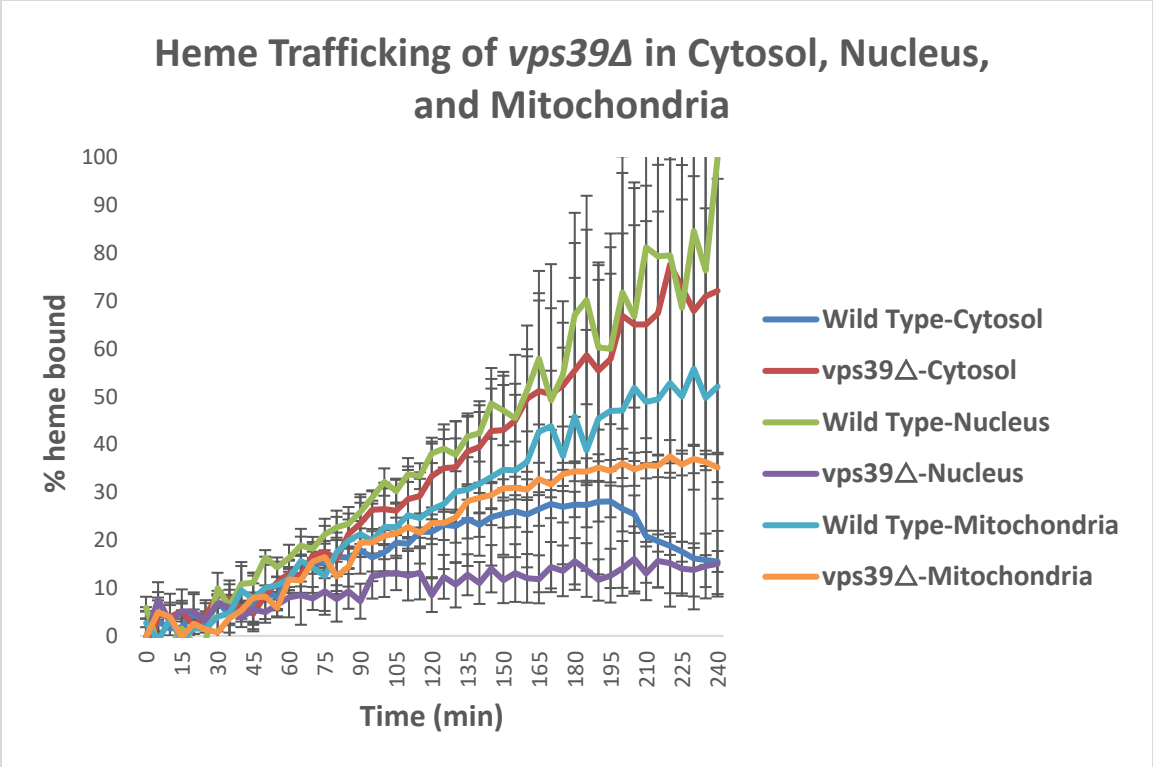


**Figure 8.** *Heme Trafficking of Vps39Δ in cytosol, nucleus, and mitochondria.* The heme saturation in Vps39 knockouts for each of the compartments was fluorescently measured over the course of four hours along with WT's under the same conditions. There was little to no trafficking to the nucleus of the Vps39Δ.

## DISCUSSION

Before heme trafficking experiments with contact point knockouts could be conducted, we wanted to learn about how heme is trafficking between organelles and discern whether the reuptake of heme occurs sequentially—implying certain compartments have higher priority for heme trafficking. Interestingly enough, we discovered that all the organelles integrate newly synthesized heme nearly simultaneously, implying the existence of parallel trafficking pathways of heme from its site of synthesis to the various cellular locations where it is incorporated. The heme re-uptake studies showed that a high degree of % heme bound can be obtained within four hours (Figure 2.), and after about 3 hours, the curve starts plateauing. Since the samples are placed in individual wells on 96-well plates, each sample is restricted to a small volume, and any duration longer than four hours causes the samples to overgrow and create bizarre fluorescent signals due to nutrient deficiency and drying out of the sample. This prevents ideal data collection beyond that point, however, for the purposes of the heme kinetics experiments with the knockouts, four hours appeared to be an ideal time because most perturbations in trafficking caused by knockouts will begin once heme re-uptake begins.

But Heme depletion takes significantly longer than heme saturation and limiting the protocol to four hours failed to show complete heme depletion. The cells are also significantly unhealthier when succinyl acetone is added and heme synthesis is inhibited, thus a 96-well plate proved to be an inadequate growth and measurement environment. The modified protocol allowed for the cells to be grown in larger flasks with better oxygenation and more nutrients for a longer period of time. By taking measurements, a steady decrease of heme was shown (Figure 3.) to sufficiently prove that the protocol worked in both saturating and depleting heme. The increase/stagnation in percent heme bound towards the end of the experiment resulted from

newly imposed environmental restrictions on the cells because for each measurement, a quantity of the sample had to be removed from the flask which caused a slow depletion of the total volume of the cell stock, while normal cell growth was still taking place. The heme depletion data also showed simultaneous depletion of heme from all three compartments (Figure 3.)

Once an understanding of heme trafficking during heme re-uptake and depletion was understood, heme kinetics experiments were performed on the five contact point mutants. Interestingly, all of the ERMES contact point mutants had the exact same trafficking perturbation: they all increased trafficking to the nucleus (Figures 4-6). Since the ERMES contact points are a direct link between the mitochondria and the nucleus (Figure 1.), we hypothesized that their removal would decrease trafficking to the nucleus, if anything at all. But, it appears as if the ERMES contact points act as negative regulator of heme mobilization to the nucleus, and their removal heighted trafficking due to disruption of the regulation.

Another potential pathway of heme mobilization could be via the vacuole, which is somewhat supported by the vCLAMPs kinetics results. The removal of Vps39 essentially inhibits heme trafficking to the nucleus. Although these results seem exciting and reveal a fair amount of new information concerning cellular heme mobilization, the vCLAMPs results did have higher than ideal level of errors and *ypt7Δ* showed results similar to those of the ERMES knockouts. Therefore, a repeat of all five contact points is necessary to ascertain the replicability of these results before any concrete conclusions can be drawn.

A recent paper highlighted the essential role of vCLAMPs in cellular viability under starvation conditions, and further elaborated the central role Vps39 plays in forming the mitochondrial-vacuolar contact point.[9] They also highlighted a vacuolar mitochondrial site that is redundant with an ER-mitochondrial site, which could explain why the *ypt7Δ* were able to retain

trafficking to the nucleus.[9] Ypt7 is also a regulator of fusion in yeast, which could affect the morphology of the knockouts, thus interfering with the kinetics studies.[10]

## CONCLUSION

The heme kinetics protocol described herein can be used to study both the saturation of cellular compartments with heme in real time and, with certain modifications, the depletion in heme from those compartments. It is also a reliable means to analyze heme trafficking via saturation of various cellular compartments. The ERMES knockouts all showed increased trafficking to the nucleus, indicating that the contact structure may negatively regulate heme trafficking to the nucleus, while one of the vCLAMPs knockouts showed increased trafficking and the other showed no trafficking to the nucleus. These experiments shed new light on the role of these contact points, especially in context of heme mobilization. They also show that heme trafficking might not be a novel and isolated pathway but incorporated into cellular structures with many diverse roles. Replications of the current experiments, and more in-depth analyses of these contact points are needed to gain a cleared understanding of each of their roles in heme trafficking.

## REFERENCES


[1]Hanna, D. A.; Harvey, R. M.; Martinez-Guzman, O; Yuan, Z; Chandrasekharan, B.; Raju, G.; Outten, F. W.; Hamza, I.; Reddi, A. R. (2016) Proceedings of the National Academy of Sciences 113 (27), 7539-7544.

[2]Chiabrando D, Vinchi F, Fiorito V,Mercurio S, Tolosano E (2014) Heme in pathophysiology: A matter of scavenging, metabolism and trafficking across cell membranes. Front Pharmacol 5:61.

[3]Sassa S (2004) Why heme needs to be degraded to iron, biliverdin IXalpha, and carbon monoxide? Antioxid Redox Signal 6(5):819–824.

[4]Ungermann, C., (2015) vCLAMPs—an intimate link between vacuoles and mitochondria. *Current Opinion in Cell Biology*, *35*, 30-36.

[5]Hönscher, C.; Mari, M.; Auffarth, K.; Bohnert, M.; Griffith, J.; Geerts, W.; van der Laan, M.; Cabrera, M.; Reggiori, F.; Ungermann, C., Cellular Metabolism Regulates Contact Sites between Vacuoles and Mitochondria. *Developmental Cell* 2014, *30* (1), 86-94.

[6]Kornmann, B.; Osman, C.; Walter, P. (2011) The conserved GTPase Gem1 regulates endoplasmic reticulum mitochondria connections . *Proceedings of the National Academy of Sciences*, *108* (34), 14151–14156.

[7]Cohen, Y.; Klug, Y. A.; Dimitrov, L.; Erez, Z.; Chuartzman, S. G.; Elinger, D.; Yofe, I.; Soliman, K.; Gärtner, J.; Thoms, S.; Schekman, R.; Elbaz-Alon, Y.; Zalckvar, E.; Schuldiner, M. (2014) Peroxisomes are juxtaposed to strategix sites on mitochondria. *Mol. BioSyst.*, *10* (7), 1742–1748.

[8]Ostrowicz, C. W.; Bröcker, C.; Ahnert, F.; Nordmann, M.; Lachmann, J.; Peplowska, K.; Perz, A.; Auffarth, K.; Engelbrecht-Vandré, S.; Ungermann, C. (2010) Defined subunit arrangement and rab interactions are required for functionality of the HOPS tethering complex. *Traffic*, *11* (10), 1334–1346.

[9]Montoro, A.; Auffarth, K.; Honscher, C.; van der Laan, M.; Frohlich, F.; Ungermann, C., Vps39 Interacts with Tom40 to Establish One of Two Functionally Distinct Vacuole-Mitochondria Contact Sites. *Developmental Cell* 2018.

[10]Balderhaar, HJ,; Arlt, H.; Ostrowicz, C.; Brocker, C.; Sundermann, F.; Brandt, R.; Babst, M.; Ungermann, C., The Rab GTPase Ypt7 is linked to retromer-mediated receptor recycling and fusion at the yeast late endosome. *Journal of Cell Science* 2010, 1;123(Pt 23):4085-94.